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A Molecular Map of T Cell Development

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Summary

Using a sensitive molecular marker for positive selection, the appearance of a particular functional TCR α chain sequence in cells from mice bearing a transgenic β chain, we address several aspects of intrathymic T cell development. First, by examining specific TCR prior to and after maturation, we demonstrate how a restricted TCR repertoire is positively selected from a highly diverse immature TCR repertoire. Second, since this molecular marker is enriched in cells progressing toward the CD4 lineage and depleted in cells progressing toward the CD8 lineage, a map of the developmental pathway of αβ thymocytes can be inferred. Third, the first cells that show clear signs of positive intrathymic selection are identified.

Introduction

The peripheral T cell population is capable of responding to a wide array of foreign antigens presented as peptides bound to self major histocompatibility complex (MHC) class I or class II molecules on the surface of antigen-presenting cells (APCs). The recognition of these MHC: peptide complexes by T cells is mediated by the T cell receptor (TCR). The integrity of the immune system relies upon the nature of the TCRs expressed by peripheral T cells. Each TCR must be capable of signaling for response to an appropriate foreign peptide:MHC ligand, but this functional signaling must be precise, and it is important that it not overlap with the effect of signaling in response to peptide:MHC complexes generated from self proteins (Kersh and Allen, 1996). When these criteria are met, pathogen-infected cells can be detected and eliminated, while surrounding, healthy cells are not directly targeted by the T cell response.

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Bone marrow–derived precursor cells must traverse a highly regulated program of intrathymic development to become mature thymocytes that then leave the thymus and establish the peripheral T cell pool (Shortman and Wu, 1996; Fehling and von Boehmer, 1997). Early stages of thymocyte development are clearly defined by several molecular events that include expression of the RAG-1 and RAG-2 gene products (Lin and Desiderio, 1993; Ferguson et al., 1994; Hoffman et al., 1996), somatic rearrangement of the T cell receptor β chain locus, expression of the coreceptor molecules CD4 and CD8, and, finally, recombination of the TCR α locus (Dudley et al., 1994; Petrie et al., 1995). Productive rearrangement of both TCR chains allows surface expression of the TCR and ensures life up to this point. Further development, however, relies in part (Tourigny et al., 1997) upon signals generated by TCR interaction with MHC: self peptide complexes (von Boehmer, 1994). Most thymocytes fail this rigorous criterion for selection and die of neglect (Janeway, 1994).

While the early events in development can be clearly defined by molecular events, later stages of development are typically defined by variations in the expression levels of cell surface proteins such as CD4, CD8, and the TCR. These molecules allow for several different intrathymic subpopulations to be distinguished. The developmental status of such populations has been, in most cases, assessed by other means including intrathymic injections of genetically marked cells and reconstitution of thymocytes in irradiated mice.

In this report, we identify a sensitive molecular marker for positive selection. This marker, which is the appearance or loss of a specific TCR α chain sequence, is used to address three issues concerning the development of TCRαβ bearing thymocytes. First, we demonstrate that the restriction of TCR usage that was apparent in the mature repertoire in "single-peptide" mice bearing a transgenic TCR β chain (Sant'Angelo et al., 1997) can also be visualized in mice expressing a wild-type intrathymic self peptide repertoire. These data support a model in which intrathymic self peptides define the specificity of the mature TCR repertoire. Second, we determine that this marker can be used in these TCR β chain transgenic mice to clearly define the developmental potential of specific thymic subpopulations. Using these data, we are able to construct a molecular map of T cell development that integrates a large body of published data and also explains some of the apparent inconsistencies between various experimental systems. Third, the detection of this particular TCR α chain proves to be a sensitive molecular marker for identifying the precise cell population in which intrathymic positive selection occurs.

Results

Peptide-Specific Responses of Nonimmunized TCR β Chain Transgenic Thymocytes and Peripheral T Cells

In this report, we have analyzed mice expressing a transgenic TCR β chain (Vβ8.2-Jβ2.6) derived from a T

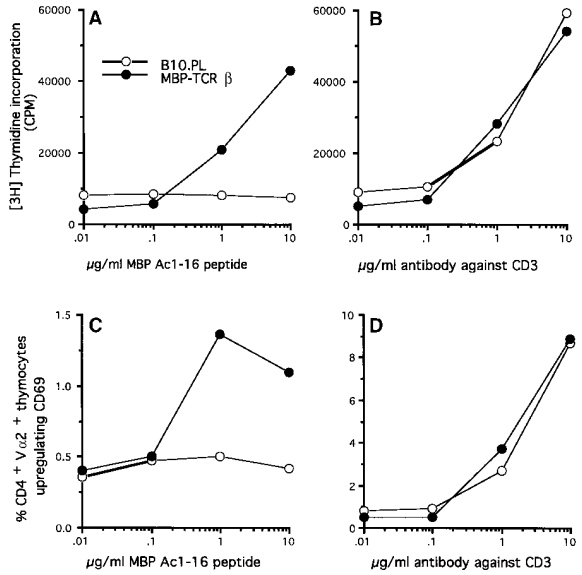

Figure 1. Both Lymphocytes and Thymocytes from an MBP-TCR β Chain Transgenic Mouse Respond to the MBP Ac1-16 Peptide

(A) MBP-β chain transgenic lymphocytes respond to MBP Ac1-16 peptide, while the control nontransgenic B10.PL lymphocytes do not respond. (B) Lymphocytes from both types of mice proliferate equally to well to anti-CD3. (C) MBP-TCR β chain transgenic $CD4^+V\alpha2^+$ thymocytes increase CD69 expression in response to MBP Ac1-16 peptide. Thymocytes were first enriched for CD4 SP cells by depletion of CD8-expressing cells. $CD4^+$, $V\alpha2^+$ thymocytes were assayed by FACS for up-regulation of CD69 as compared to cells prepared by the same methods; however, incubated without peptide (D), $CD4^+$, $V\alpha2^+$ thymocytes from both a nontransgenic mouse and an MBP-TCR β chain transgenic mouse up-regulate CD69 in response to anti-CD3 stimulation as compared to cells not treated with anti-CD3.

cell hybrid produced using cells from a B10.PL ($H\text{-}2^u$) mouse and responsive to mouse myelin basic protein (MBP) peptide, Ac1-16, presented by $I\text{-}A^u$ (Goverman et al., 1993). This β chain is paired in this T hybridoma with a TCR α chain using the Vα2.3 and Jα11 gene segments. The relative frequency of T cells bearing MBP:$I\text{-}A^u$ reactive TCR in the total lymphocyte population was estimated by culturing lymphocytes from unimmunized mice carrying this TCR β chain transgene (MBP-TCR β mice) and from a nontransgenic B10.PL mouse with the Ac1-16 MBP peptide. As shown in Figure 1A, lymphocytes from the β chain transgenic mouse responded strongly to the added peptide, while nontransgenic lymphocytes did not respond. Cells from both mice were equally responsive to antibody against CD3 (Figure 1B). This primary T cell response suggested that the naive TCR repertoire in the MBP-TCR β mice was highly enriched for cells bearing TCRs that are able to recognize the Ac1-16:$I\text{-}A^u$ complex.

To determine if there was also a high frequency of antigen-specific thymocytes in the MBP-β chain transgenic mice, we compared the percentage of thymocytes that were activated with anti-CD3 antibody to the percentage of thymocytes that were activated by the MBP Ac1-16 peptide. CD4 single-positive (SP) thymocytes from a MBP-TCR β mouse and a transgene negative littermate were isolated and then incubated with T-depleted, mitomycin C–treated B10.PL splenocytes plus either anti-CD3 antibody or MBP Ac1-16 peptide for 6 hr. Following incubation, the cells were stained with antibodies against CD4, Vα2, and the early activation marker, CD69. The percentage of $CD4^+$, $V\alpha2^+$ cells that had up-regulated CD69 was determined by FACS.

Only MBP-TCR β chain transgenic thymocytes were activated by the MBP Ac1-16 peptide (Figure 1C) as assessed by up-regulation of CD69. Thymocytes from both the transgenic and nontransgenic mice were found to increase CD69 expression following incubation with anti-CD3 (Figure 1D). More than 1% of the $CD4^+$, $V\alpha2^+$ thymocytes increased their CD69 expression as a result of incubation with peptide as compared to 10% of the $CD4^+$, $V\alpha2^+$ cells incubated with anti-CD3. Together, the lymphocyte and thymocyte activation data confirm that a substantial percentage of the total lymphocyte and thymocyte population in mice transgenic for the β chain of this MBP-specific hybrid recognize the MBP peptide.

Dominant Use of the Parental Amino Acid CDR3α Sequence in Vα2-Jα11 CD4 T Cells and Thymocytes from MBP-β Chain Transgenic Mice

We next wanted to determine the frequency of Vα2.3-Jα11 T cells in MBP-TCR β mice that had TCR identical in amino acid sequence to the original T cell clone, as this would suggest specific recognition of both the MBP:$I\text{-}A^u$ complex and a limited set of other self-peptide $I\text{-}A^u$ molecules during intrathymic selection. To do this, we sorted cells that expressed Vα2 and CD4 from both the periphery and thymus of two MBP-TCR β mice and extracted the genomic DNA. The highly restricted use of Vα2.3-Jα11 TCR α chains in hybridomas raised in nontransgenic B10.PL mice specific for MBP suggested that most of the Ac1-16:$I\text{-}A^u$-specific T cells in the MBP-TCR β mouse would use the same TCR gene segments (Urban et al., 1988). Therefore, we amplified TCR α chains by PCR from the sorted cells with primers specific for Vα2.3 and Jα11. The product, which encompassed the TCR CDR3α region, was cloned and sequenced as previously described (Sant'Angelo et al., 1997). The sequences from one such experiment are shown in Figures 2A and 2B. The CD4 SP thymocyte DNA prepared this way (Figure 2A) was dominated by this particular CDR3α sequence, as were the sequences from the peripheral CD4 T cells (Figure 2B). Approximately 70% of the analyzed thymocyte and lymphocyte sequences from the combined results of two mice express the parental CDR3α sequence (Figure 2C). Importantly, the parental sequence was not seen in the $CD8^+$ thymocytes (Figure 2D), demonstrating that this sequence is rarely if ever selected on MHC class I in these animals.

A Highly Restricted TCR Repertoire Is Selected from a Diverse Preselection Repertoire

We next examined whether this enrichment for antigen-specific TCR was due to intrathymic positive selection. Thymocytes from an MBP-TCR β mouse were sorted using antibodies to CD4, CD8, and TCR (Figures 3A and 3B) into a primarily $CD4^+TCR^{lo}$ preselection population

A

	Thymus CD4+Vα 2+								#
	C	A	A	S	A	N	S	G	
Parental	TGT	GCA	GCA	AGT	GCA	AAT	TCT	GGG	8/15
	C	A	A	S	E	N	S	G	
	TGT	GCA	GCA	AGT	GAA	AAT	TCT	GGG	2/15
	TGT	GCA	GCA	AGC	GAA	AAT	TCT	GGG	1/15
	C	A	A	S	D	N	S	G	
	TGT	GCA	GCA	AGT	GAC	AAT	TCT	GGG	1/15
	C	A	A	S	P	N	S	G	
	TGT	GCA	GCA	AGT	CCA	AAT	TCT	GGG	1/15
	C	A	A	S	A R	N	S	G	
	TGT	GCA	GCA	AGT	GCA AGG	AAT	TCT	GGG	1/15
	C	A	A	M	D		S	G	
	TGT	GCA	GCA	ATG	GAT		TCT	GGG	1/15

B

	Lymph node CD4+Vα 2+								#
	C	A	A	S	A	N	S	G	
Parental	TGT	GCA	GCA	AGT	GCA	AAT	TCT	GGG	10/16
	TGT	GCA	GCA	AGT	GCT	AAT	TCT	GGG	1/16
	TGT	GCA	GCA	AGT	GCG	AAT	TCT	GGG	1/16
	C	A	A	S	L	D	S	G	
	TGT	GCA	GCA	AGT	TTG	GAT	TCT	GGG	1/16
	C	A	A	S	E	N	S	G	
	TGT	GCA	GCA	AGT	GAA	AAT	TCT	GGG	1/16
	C	A	A	S	P	N	S	G	
	TGT	GCA	GCA	AGT	CCC	AAT	TCT	GGG	1/16
	C	A	A	G	V	N	S	G	
	TGT	GCA	GCA	GGT	GTA	AAT	TCT	GGG	1/16

C

Totals (2 Mice)	Thymus	Periphery
Parental	66% (19/29)	75% (44/59)
Other	34% (10/29)	25% (15/59)

D

	Thymus CD8+Vα 2+								#
	C	A	A	S	A	N	S	G	
Wild type	TGT	GCA	GCA	AGT	GCA	AAT	TCT	GGG	0/14
	C	A	A	S	A	G	S	G	
	TGT	GCA	GCA	AGT	GCT	GGT	TCT	GGG	3/14
	C	A	G	S	L	G	S	G	
	TGT	GCA	GGA	AGT	CTG	GGA	TCT	GGG	1/14
	C	A	A	S	E	A	G		
	TGT	GCA	GCA	AGT	GAG	GCT	GGG		2/14
	C	A	A		I	N	S	G	
	TGT	GCA	GCA		ATC	AAT	TCT	GGG	1/14
Out of frame									7/14

Figure 2. The Parental CDR3 α Segment Dominates Vα2.3 TCRs from Thymocytes and Lymphocytes

TCR Vα2.3-Jα11 CDR3α chain sequences obtained from $CD4^+$ $CD8^-TCR^{hi}$ thymocytes (A) and $CD4^+$ peripheral T cells (B) from a MBP-TCRβ chain transgenic mouse. (C) Greater than 70% of the sequences obtained from $CD4^+$ $Vα2^+$ T cells from two mice have the parental CDR3 sequence, (D) while none of the sequences from the $CD8^+$ $Vα2^+$ thymocytes were parental.

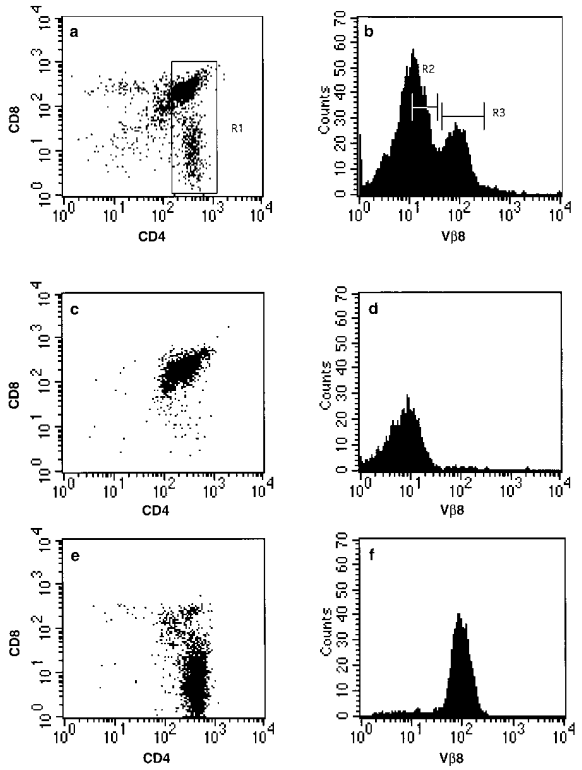

Figure 3. Separation of the Thymus of Mice Transgenic for a Vβ8.2 Transgene into TCR^{lo} and TCR^{hi} $CD4^+CD8^-$ Thymocytes

T cells that have been either positively selected (TCR^{hi}) or T cells not yet selected (TCR^{lo}) were obtained from the thymus of an MBP-TCRβ chain transgenic mouse by staining with antibodies specific for CD4, CD8, and Vβ8 followed by separation by fluorescence-activated cell sorting (FACS). By gating on the $CD4^+$ (A) and collecting cells that expressed the TCR transgene-encoded Vβ8 at high or low levels (B), we obtained the populations shown in C–F. TCR^{lo} thymocytes are predominantly $CD4^+CD8^+$ (C and D), while TCR^{hi} thymocytes are largely $CD4^+CD8^-$ (E and F).

(Figures 3C and 3D) or a primarily $CD4^+TCR^{high}$ postselection population (Figures 3E and 3F). Genomic DNA was prepared from these sorted thymocytes, PCR amplified, cloned, and sequenced as above. The TCR^{lo} thymocytes displayed almost complete heterogeneity in Vα2.3-Jα11 CDR3 sequence (Figure 4A), while in sharp contrast over 60% of the Vα2.3-Jα11 chain CDR3 sequences obtained from the positively selected TCR^{hi} thymocytes were identical to the parental clone at the amino acid level (Figure 4B). This demonstrates that the restricted Vα2.3-Jα11 TCR repertoire detected in the MBP-TCR β mice is selected from a very diverse set of TCR chains. We also examined the frequency of out-of-frame Vα2.3-Jα11 joins. In our previous sorts (Figure 2), we used an antibody against Vα2 and, therefore, thymocytes bearing out-of-frame joins were not frequently collected. The sharp decrease in the percentage of out-of-frame joins suggests positive selection for the parental TCR because negative selection cannot eliminate out-of-frame sequences as they are not expressed at the cell surface. As before, the parental TCR α chain was never found in CD8 SP thymocytes (data not shown). These data demonstrate that the enrichment for TCR that recognize the Ac1-16 peptide is a result of positive selection occurring in the thymus. Since the restriction in TCR sequence occurs within the CDR3α segment, an important MHC class II bound peptide contact point (Jorgensen et al., 1992; Sant'Angelo et al., 1996), it is likely that intrathymic self peptides are having a significant impact on this selection event. The common nucleotide usage seen in many of the parental sequences is expected since this TCR α chain can result from a direct Vα to Jα join without the need for gain or loss of nucleotides. PCR reactions were performed simultaneously when possible. Any potential PCR bias toward a specific sequence would have been detected in sequences from both pre- and postselection populations. Such a skewing never occurred in these experiments or in previous work (Sant'Angelo et al., 1997).

$CD4^{lo}CD8^{lo}CD69^+$ Thymocytes Already Have TCR α Chain Usage Characteristic of a Positively Selected Population

Having demonstrated that we could visualize positive selection of a population of thymocytes using this molecular marker, we next sought to establish precisely

A

		C	A	A	S		A		N	S	G	
Germline	TGT	GCA	GCA	AGT	G			CA	AAT	TCT	GGG	
						TCR^{lo} Thymocytes						#
Parental	C	A	A	S		A		N	S	G		
	TGT	GCA	GCA	AGT		GCA		AAC	TCT	GGG		1/56
	TGT	GCA	GCA	AGC		GCA		AAT	TCT	GGG		3/56
	C	A	A	S		E		N	S	G		
	TGT	GCA	GCA	AGT		GAA		AAT	TCT	GGG		1/56
	C	A	A	S		V		N	S	G		
	TGT	GCA	GCA	AGT		GTA		AAT	TCT	GGG		1/56
	C	A	A	S		P		N	S	G		
	TGT	GCA	GCA	AGT		CCA		AAT	TCT	GGG		1/56
	TGT	GCA	GCA	AGC		CCA		AAT	TCT	GGG		1/56
	C	A	A	S		T		N	S	G		
	TGT	GCA	GCA	AGT		ACA		AAT	TCT	GGG		1/56
	C	A	A			R		N	S	G		
	TGT	GCA	GCA			AGG		AAT	TCT	GGG		1/56
	TGT	GCA	GCA			AGA		AAT	TCT	GGG		1/56
	C	A	A		R	R		N	S	G		
	TGT	GCA	GCA		AGG	AGA		AAT	TCT	GGG		6/56
	C	A	A	S		E	D		S	G		
	TGT	GCA	GCA	AGT		GAA	GAT		TCT	GGG		1/56
	C	A	A	S		G	D		S	G		
	TGT	GCA	GCA	AGT		GGG	GAT		TCT	GGG		1/56
	C	A	A	S		G	G		S	G		
	TGT	GCA	GCA	AGT		GGA	GGT		TCT	GGG		1/56
	C	A	T	S		E	S		S	G		
	TGT	GCA	ACA	AGT		GAA	AGT		TCT	GGG		1/56
	C	A	A	S		N	I	L		G		
	TGT	GCA	GCA	AGT		AAC	ATA	CTC		GGG		1/56
	C	A	A		A	K	Q	S		G		
	TGT	GCA	GCA		GCA	AAA	CAA	TCA		GGG		1/56
	C	A	A	S	G	G		N	S	G		
	TGT	GCA	GCA	AGT	GGA	GGA		AAT	TCT	GGG		1/56
	C	A	A	G	S	V	L	S		G		
	TGT	GCA	GCA	GGC	TCT	GTT	CTT	TCA		GGG		1/56
	C	A	A			R	D		S	G		
	TGT	GCA	GCA			AGG	GAT		TCT	GGG		1/56
	C	A	A	S			V		S	G		
	TGT	GCA	GCA	AGT			GTT		TCT	GGG		1/56
	C	A				T	S	A		G		
	TGT	GCA				ACA	AGT	GCA		GGG		1/56
	S	A	A			S	A			G		
	AGT	GCA	GCA			AGT	GCA			GGG		1/56
	C	A	A			K	W			G		
	TGT	GCA	GCT			AAG	TGG			GGG		1/56
Out-of-frame												26/56

B

						TCR^{hi} Thymocytes						
Parental	C	A	A	S		A		N	S	G		
	TGT	GCA	GCA	AGT		GCA		AAT	TCT	GGG		12/23
	TGT	GCA	GCA	AGC		GCA		AAT	TCT	GGG		1/23
	TGT	GCA	GCA	AGT		GCG		AAT	TCT	GGG		1/23
	C	A	A	S		T		N	S	G		
	TGT	GCA	GCA	AGT		ACG		AAT	TCT	GGG		1/23
	C	A	A	S		P				G		
	TGT	GCA	GCA	AGT		CCC				GGG		1/23
	C	A	A		G	A		N	S	G		
	TGT	GCA	GCA		GGT	GCA		AAT	TCT	GGG		1/23
	C	A	A	S						G		
	TGT	GCA	GCA	TCT						GGG		1/23
	C	A		T	R	R	R		S	G		
	TGT	GCA		ACA	AGA	AGG	CGT		TCT	GGG		1/23
Out-of-frame												4/23

Figure 4. Most CD4$^+$Vα2 TCR^{hi} Thymocytes Use the Same CDR3α Segment

The majority of the amino acid sequences of Vα2.3-Jα11 CDR3α segments obtained from TCR^{lo} cells (as shown in Figures 3C and 3D) are very diverse, with few containing the parental clone sequence (7%, 4 parental sequences/56 total sequences) and many (46%, 26/56) out-of-frame sequences. CD4$^+$TCR^{hi} thymocytes (from Figures 3E and 3F), however, express many Vα2-Jα11 CDR3α amino acid sequences (61%, 14/23) that are the same as the parental T cell hybrid and few (17%, 4/23) out-of-frame sequences. #, number of sequences of this type/total number of sequences.

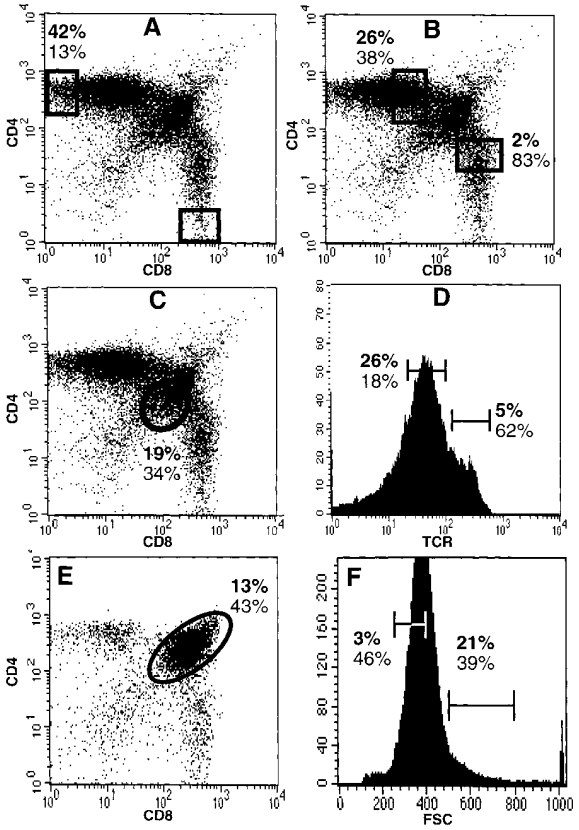

Figure 5. The Developmental Status of Intrathymic Subpopulations Is Revealed by TCR α Chain Analysis

The developmental status of individual thymic subpopulations is revealed by the percentage of parental sequences (bold type) and the percentage of out-of-frame sequences (plain type). The marked populations were separated by FACS followed by DNA extraction, PCR, cloning and sequencing.

(A) The CD4$^+$CD8$^-$CD69$^+$TCR^{hi} population has a high frequency of the parental sequence and a low frequency of out-of-frame joins as compared to the CD4$^-$CD8$^+$CD69$^+$TCR^{hi}, which has none of the parental sequences and a high percentage of out-of-frame sequences. These differences in sequence usage clearly mark these mature populations as CD4 SP and CD8 SP, respectively.

(B) The CD4$^+$CD8^{lo}CD69$^+$TCR$^{int/hi}$ has α chain usage characteristic of a mix of CD4 and CD8 cells, while the CD4^{lo}CD8$^+$CD69$^+$TCR$^{int/hi}$ is depleted of the MHC class II cells using this α chain sequence.

(C) The CD4^{lo}CD8^{lo}CD69$^+$TCR$^{int/hi}$ population is already enriched for the parental TCR α chain.

(D) When the CD4^{lo}CD8^{lo}CD69$^+$TCR$^{int/hi}$ population is further separated based on TCR level, it becomes clear that the CD4^{lo}CD8^{lo}CD69$^+$TCR^{int} cells are a mix of cell types while the CD4^{lo}CD8^{lo}CD69$^+$TCR^{hi} are depleted of cells bearing the MHC class II restricted TCR.

(E) The parental TCR α chain sequence (bold type) can frequently be found in CD4$^+$CD8$^+$CD69$^-$ thymocytes. (F) After further separation of this population into small and enlarged cells, however, it can be seen that most of the parental sequences are found in the large cells. This enrichment of thymocytes bearing MHC class II–specific TCR demonstrates that positively selected CD4$^+$ T cells first appear after the transition from a small to large CD4$^+$CD8$^+$TCR^{lo}CD69$^-$ phenotype. The frequency of out-of-frame Vα2.3-Jα11 sequences (plain text) does not significantly change.

when positive selection occurs. Delineation of the various thymic subpopulations was based on a synthesis of recent data in which the progression of the developing thymocyte was inferred, in part, by following the complex regulation of TCR, CD4, and CD8 along with the early activation marker CD69 (Lucas and Germain, 1996). The bulk of double-positive (DP) TCR$^{-/lo}$ cells do not express CD69. Expression of CD69 is also absent in TCR α chain–deficient mice as well as in mice having both MHC class I and class II genes inactivated by gene targeting (Lucas and Germain, 1996). We therefore began our search by collecting the various CD69$^+$ transitional subpopulations.

We first collected mature CD69$^+$ thymocytes that express high levels of TCR together with either CD4 or CD8. Sequence evaluation of TCR Vα2 chains in these two mature populations (Figure 5A) clearly demonstrates the distinction between CD4 thymocytes that

Table 1. Number of Each Sequence Type Obtained from Individual Cell Sorts

Subpopulation	Sort 1	Sort 2	Sort 3	Sort 4	Totals	Parental	Out-of-Frame
$CD4^+CD8^+CD69^-$	1/19/16	1/13/13	0/6/10		2/38/39	3%	49%
Double Positive Small	(36)	(27)	(16)		(79)		
$CD4^+CD8^+CD69^-$	6/12/17	8/10/8	4/13/9		18/35/34	21%	39%
Double Positive Large	(35)	(26)	(26)		(87)		
$CD4^{lo}CD8^{lo}CD69^+$	12/21/13	9/24/4	7/16/3		28/61/20	26%	18%
Double Dull TCR^{int}	(46)	(37)	(26)		(109)		
$CD4^+CD8^{lo}CD69^+$	7/13/12	9/10/8	3/4/2	8/11/17	27/38/39	25%	38%
$TCR^{int/hi}$	(32)	(27)	(9)	(36)	(109)		
$CD4^+CD8^-CD69^+$	7/16/5	13/11/6	18/14/1		38/41/12	42%	13%
Single Positive	(28)	(30)	(33)		(91)		
$CD4^+$ Lymphocytes	6/10/2	16/7/0	16/13/3		38/30/5	52%	7%
	(18)	(23)	(32)		(73)		
$CD4^{lo}CD8^{lo}CD69^+$		2/10/11	1/9/25		3/19/36	5%	62%
Double Dull TCR^{hi}		(23)	(35)		(58)		
$CD4^{lo}CD8^+CD69^+$		1/6/22	0/3/28		1/9/50	2%	83%
$TCR^{int/hi}$		(29)	(31)		(60)		
$CD4^+CD8^+CD69^+$	0/3/9	0/4/15			0/7/24	0%	77%
Single Positive	(12)	(19)			(31)		
$CD8^+$ Lymphocytes	0/0/13	0/0/8			0/0/21	0%	100%
	(13)	(8)			(21)		

The number of times each sequence type was found in individual experiments is shown as # parental/# nonparental/# out-of-frame. The totaled numbers as well as the percentages used in Figures 6 and 7 are also shown. All cell sorts were repeated at least two times. Closely related transitional populations (i.e., $CD4^+CD8^{lo}TCR^{int/hi}$ and $CD4^+CD8^-TCR^{hi}$ were sorted from the same mouse.

have been positively selected on MHC class II and CD8 thymocytes that are positively selected on MHC class I. The $CD4^+CD8^-CD69^+$ single-positive cells using a $V\alpha2$-$J\alpha11$ TCR α chain have a high percentage of sequences that are identical at the amino acid level to the TCR α chain of the MBP-reactive parental clone (percent parental) as well as a very low percentage of out-of-frame sequences (percent out-of-frame). This is clearly distinct from the mature $CD4^-CD8^+CD69^+$ SP cells in which there are no parental α chains and a very high frequency of $V\alpha2$-$J\alpha11$ out-of-frame joins. These results were highly reproducible, as shown in Table 1.

The transitional populations, comprising the $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ and the $CD4^{lo}CD8^+CD69^+TCR^{int/hi}$ are also distinctive (Figure 5B; Table 1). $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ cells have significantly fewer parental sequences and many more out-of-frame joins, which suggests that this population is a mixture of MHC class I and MHC class II restricted cells. The counterpart $CD4^{lo}CD8^+CD69^+TCR^{int/hi}$ population (Figure 5B; Table 1), however, has mostly out-of-frame sequences similar to those found in the $CD4^-CD8^+CD69^+$ SP thymocytes. This strongly suggests that cells within this later transitional population are committed to becoming $CD4^-CD8^+CD69^+$ SP, MHC class I restricted T cells (van Meerwijk and Germain, 1993).

Thymocytes transit through a $CD4^{lo}CD8^{lo}CD69^+TCR^{int/hi}$ "double dull" population (Figure 5C) that has been suggested to contain the immediate precursors of the $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ population (Lucas and Germain, 1996). In support of this relationship, the double dull population revealed a frequency of parental α chains not substantially different from that in the succeeding $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ populations. The double dull population, however, can be further subdivided into cells expressing either high or intermediate TCR levels (Figure 5D). The TCR^{hi} cells in the double dull population are predicted to be cells differentiating to $CD4^-CD8^+CD69^+$ SP after passing through the $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ stage (van Meerwijk and Germain, 1993; Lucas and Germain, 1996). That these cells are destined, at this point, to become CD8 SP is fully consistent with the very low frequency of parental α chains and the high frequency of out-of-frame sequences typical of mature CD8 cells (Figure 5D; Table 1). This is in contrast to the double dull cells that are TCR^{int}. The percentage of each type of sequence in this subpopulation is very characteristic of a mixed MHC class I and MHC class II restricted population (Figure 5D; Table 1). It is clear, however, that an enrichment for the MHC class II restricted TCR sequence has occurred. Therefore, while the final sorting of the cells into the different mature lineages has yet to occur, this $CD4^{lo}CD8^{lo}CD69^+TCR^{int}$ population has clearly already undergone positive selection.

Positive Selection Appears to Occur during a Transition from Small to Enlarged $CD4^+CD8^+CD69^-$ Thymocytes

Having analyzed all of the previously characterized populations of $CD69^+$ thymocytes and found evidence of positive selection in all of them, we concluded that initiation of positive selection must occur prior to the upregulation of CD69 or the TCR. We therefore electronically gated on $CD69^-$ thymocytes and then collected the $CD4^+CD8^+$ population. Only 13% of the sequences from this population showed the parental α chain join (Figure 5E). Indeed, in our original sorts (Figures 3 and 4) of TCR^{lo} cells, we had also found an unexplained "background" of 7% of the sequences having the parental α chain. Having no other surface marker to distinguish among these $CD4^+CD8^+TCR^{lo}CD69^-$ cells, we turned to separation based on differences in the size of the cells. As shown in Figure 5F and Table 1, this DP subpopulation can be divided into small cells and larger cells. Sequence analysis of TCR α chains from these two populations revealed that the frequency of parental-type

Vα2 chains in the small $CD4^+CD8^+CD69^-$ cells dropped to only 2.5% (2/79 total sequences; Figure 5F; Table 1), while the frequency within the enlarged population increased to 21% (18/87 sequences; Figure 5F; Table 1). Clearly, an enrichment for the parental TCR α chain has occurred in the TCR^{low} $CD69^-CD4^+CD8^+$ enlarged cells as compared to the small cells.

Discussion

Previously, we and others have demonstrated that T cell hybridomas derived from peptide-immunized TCR β chain transgenic mice are highly restricted in their TCR α chain usage (Jorgensen et al., 1992; Sant'Angelo et al., 1996). For example, in our studies, 22 of 22 T cell hybridomas raised against the antigenic peptide that stimulated the T cell clone from which the β chain was derived had TCR α chain sequences that were identical at the amino acid level (Sant'Angelo et al., 1996). Later, we demonstrated that this restriction in TCR usage was already apparent in the naive TCR repertoire and, therefore, was not simply a clonal expansion of a few T cells as a result of the peptide immunization (Sant'Angelo et al., 1997). These data led us to suggest that positive selection on intrathymic self peptides has a strong impact on the specificity of the mature TCR repertoire. To establish the role of self peptides in intrathymic selection, we demonstrated that alteration of the intrathymic self peptide repertoire, achieved by elimination of the peptide exchange protein H2-Ma, had a dramatic impact on the structure and specificity of the mature TCR repertoire (Sant'Angelo et al., 1997).

In this report, using a different TCR β chain transgenic mouse, we again demonstrate the significant impact that positive selection has on the specificity of the mature TCR repertoire. However, in this system, the restriction in TCR α chain usage is readily apparent in mice expressing a wild-type repertoire of intrathymic self peptides. A comparison of the α chain CDR3 loops of TCR from immature thymocytes to the same region of TCR expressed by mature thymocytes clearly demonstrates how a restricted mature repertoire is selected from a highly diverse repertoire of immature thymocytes. Previous work has demonstrated that CDR3α makes an important contact with the MHC class II bound peptide (Jorgensen et al., 1992; Sant'Angelo et al., 1996) and, therefore, suggests that this restriction in TCR usage is explained by the necessity for an interaction with an intrathymic self-peptide or a limited, structurally related set of such peptides.

Our data allow for the development of a model that integrates and explains numerous reports concerning αβ T cell development. This model, as shown in Figure 6, suggests that $CD4^+CD8^+CD69^-$ TCR^{lo} enlarged cells have already initiated TCRαβ positive selection. Since it has been shown that small cortical thymocytes can undergo positive selection (Lundberg and Shortman, 1994; Swat et al., 1994), we suggest that these positively selected enlarged $CD4^+CD8^+CD69^-$ TCR^{lo} cells arise directly from the small, resting double-positive thymocytes as shown in Figure 6. These cells can live for up to 4 days allowing time for TCR α chain recombination

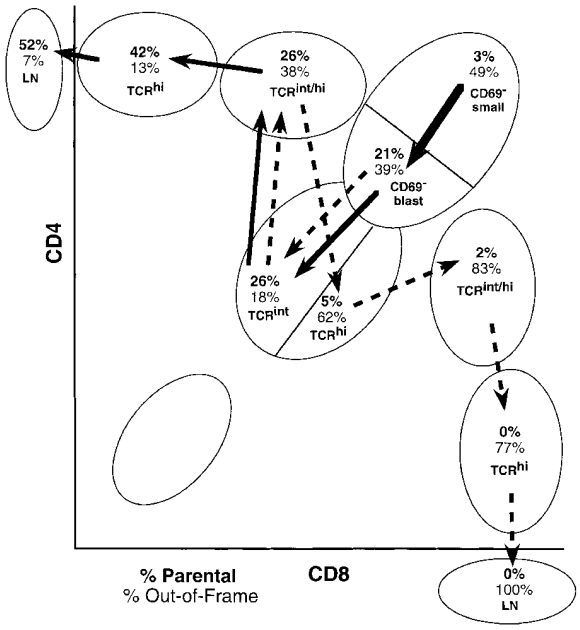

Figure 6. A Molecular Map of T Cell Development

A summary of data presented here provides a map of thymocyte development. Numbers represent the percentage of parental TCR α chain sequences (bold text) as compared to the number of out-of-frame sequences (plain text). Cells are believed to develop as indicated by arrows, with several populations carrying two subpopulations with differences in their developmental potential. We suggest that the primary positive selection event occurs during the transition from the small to large $CD69^-CD4^+CD8^+TCR^{lo}$ phenotype. Bold arrow, positive selection; intermediate solid lines, CD4 lineage development; dashed lines, CD8 lineage development. See text for details.

to occur. If an appropriate TCR is produced, positive selection occurs, and the small cells enter the enlarged population. We do not believe that this enlargement necessarily leads to cell division, but this question has not been addressed.

Recognition of MHC by the TCR results in down regulation of both the CD4 and the CD8 coreceptor molecules resulting in the $CD4^{lo}CD8^{lo}CD69^+TCR^{int/hi}$ double dull subpopulation (Lucas and Germain, 1996). Sequence analysis clearly establishes this subpopulation as post-positive selection. Furthermore, it is clear that the double dull cells consist of two distinct populations. The TCR^{int} cells consist of α chain sequences consistent with this population having both CD4 and CD8 precursor cells, whereas the double dull TCR^{hi} population has been clearly depleted of the MHC class II restricted sequence and, therefore, represents a CD8 precursor population (van Meerwijk and Germain, 1993).

Faster reexpression of CD4 relative to CD8 appears to give rise to $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ cells that are believed to be a mix of both CD4 and CD8 committed cells (Suzuki et al., 1995; Lucas and Germain, 1996), while $CD4^{lo}CD8^+CD69^+TCR^{int/hi}$ cells have been suggested to contain only CD8 precursors (van Meerwijk and Germain, 1993; Lundberg et al., 1995). Indeed, intrathymic injections of sorted cells, thymic organ culture

experiments, and bcl-2 transgenic thymocyte cell cultures have provided data consistent with these predictions (Kydd et al., 1995; Lundberg et al., 1995; Barthlott et al., 1997). Our sequence data now confirm that while the $CD4^+CD8^{lo}CD69^+TCR^{int/hi}$ appear to be a mix of lineage types, the $CD4^{lo}CD8^+CD69^+TCR^{int/hi}$ are depleted of MHC class II restricted cells. The subsequent maturation to true TCR^{hi} SP results in a CD4 SP population that is clearly distinct from the immediate precursor $CD4^+CD8^{lo}$ population, while the CD8 SP and the $CD4^{lo}CD8^+$ were very similar.

Finally, our data allow for a comparison of the intrathymic SP TCR repertoire with that found in the periphery. A comparison of the sequence percentages found in the CD4 SP to those found in peripheral CD4 T cells suggests a further post-thymic increase in the percentage of parental sequences (see also Figures 1 and 2). Furthermore, a striking difference becomes apparent when the CD8 SP thymocytes are compared to the peripheral CD8 population. In the SP CD8 cells, 23% of the TCR α chains using Vα2.3-Jα11 are in-frame sequences that are different from the parental MHC class II restricted α chain. Surprisingly, these in-frame Vα2.3-Jα11 are completely depleted from peripheral CD8 T cells. These data suggest an additional intrathymic or peripheral selection event subsequent to the SP stage. Perhaps this change in TCR repertoire is related to the prolonged thymic retention of newly generated SP thymocytes (Egerton et al., 1990; Rooke et al., 1997) or a late intrathymyic expansion of SP cells (Penit and Vasseur, 1997).

Utilizing a sensitive molecular approach, we have been able to firmly establish that positive selection occurs at a precise stage early in thymocyte differentiation. In addition, as summarized in Figure 6, our data allow the progression of thymocyte development to be tracked though various thymic subpopulations by measuring a molecular marker. Importantly, it is likely that the conclusions drawn from examining TCR β chain transgenic mice relate to the wild-type situation since all of the examined thymocyte subpopulations are also present in normal mice. The determination that positive selection occurs at this early stage in thymocyte development raises important questions about the role of the well characterized transitional states between positive selection and maturity (SP TCR^{hi}). It is likely that these stages represent further checkpoints that mediate tolerance (Guidos et al., 1990; Page et al., 1996) and further define lineage choice (Petrie et al., 1993; Lucas et al., 1995; Suzuki et al., 1995; Akashi and Weissman, 1996; Punt et al., 1996). Knowing when positive selection occurs and how thymocytes traffic through various developmental stages should allow for further understanding of these complex processes.

Experimental Procedures

Proliferation and Thymocyte Activation

Lymph node proliferation assays were performed with 5×10^5 cells per well in round-bottom 96-well plates. Proliferative responses were measured by adding 1 μCi ^{3}H-TdR per well on day 2 of a 3-day culture.

Thymocyte activation was determined by up-regulation of CD69 (PharMingen) as detected by FACS. Thymic single-cell suspensions were depleted of $CD8^+$ cells by incubation with anti-CD8 antibodies (TIB-105 and TIB-210) followed by incubation with magnetic beads coated with rat IgG, which mediates separation. T-depleted splenocytes were prepared by incubating a single cell suspension of a B10.PL spleen with antibodies to CD4 (GK1.5), CD8 (TIB105), and Thy1 (Y19) followed by rabbit complement and mitomycin C treatment.

Thymocytes (5×10^5) and splenocytes (1×10^6) were incubated in round-bottom plates with either anti-CD3, peptide (MBP Ac1-16), or just APCs at 37°C for 6 hr. Cells were then washed and incubated with anti-CD4 (670), anti-CD69 (PE), and anti-Vα2 (FITC) for 30 min on ice, washed, and analyzed by FACS. Events (200,000) were collected for each sample. For both lymphocyte and thymocyte assays, each data point was done in triplicate and averaged. The experiments were repeated at least once with similar results.

FACS Separations

For cell sorts, single cell suspensions of the thymus or lymph nodes were incubated on ice with the appropriate antibodies for 30 min, washed, and sorted on a FACStar Plus (Becton-Dickinson). For three-color cell sorting, staining was done with CD4-670 (Sigma), CD8-PE, and either Vα2 or Vβ8 (PharMingen). Four-color sorts were done with CD4-670, CD8-FITC, TCR-PE, and CD69-biotin revealed by Texas red streptavidin. Separation of closely related populations was always done simultaneously. Therefore, potentially contaminating cells, such as SP cells within the DP sort, would be extremely likely to be collected into both populations. For example, $CD4^+CD8^+CD69^-$ thymocytes were collected by first electronically gating on $CD69^-$ cells, followed by gating on the DP cells, and then separation based on size.

PCR Amplification

To analyze DNA prepared from the MBP-TCRβ chain transgenic mice, the following primers were used: Vα2 (VαMBP.Primary: ACTG CAGTTATGAGAACAGTG) and Jα11(JαMBP.Primary: CTCCTTTGCT TGCATCTCCTG). 30 cycles of PCR were done using 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1.5 min. A secondary PCR was then performed to discriminate between various Vα2 family members using 5 μl of the primary PCR reaction and primers close to the CDR3 region (VαMBP.RFLP: TGCCAGAATTCAAAGCTCTCCTTGCA CATTG; JαMBP.RFLP: CGATCTGGATCCGTTCCAAACCTCTGGTAAG TCC). 35 PCR cycles were done as above. All PCR reactions were done simultaneously whenever possible, and extensive controls were done to detect contamination.

Genomic DNA Preparation

DNA extraction was done as in Sant'Angelo et al. (1997) using DNazol (GIBCO-BRL).

Cloning and Sequencing

Cloning and sequencing were done essentially as in Sant'Angelo et al. (1997). In brief, PCR products, which ranged in size from 100 to 150 bp, were visualized on gels made from 0.7% agarose (BRL) and 1.3% Synergel (Diversified Biotech, Boston, MA). PCR products were directly cloned into the TopoTA vector (Invitrogen). Prior to cloning, the PCR products were purified and reincubated with Taq polymerase and dNTPs to ensure efficient addition of the 3′ adenosine nucleotides necessary for TA cloning. Individual bacterial colonies were picked, and the inserts were PCR-amplified with the M13(-20) and M13 reverse primers. Blue/white color selection of the colonies mediated by β-galactosidase was not utilized to avoid potential false blue colonies due to "read-through" of the insert. This avoided potentially skewing the results toward in-frame Vα segments. These resulting PCR products were analyzed on 0.7% agarose, 1.3% Synergel gels. All PCR products from plasmids containing inserts were purified (QIAquick PCR purification kit) and sequenced with the T7 primer using the Taq DyeDeoxy Terminator Sequencing Kit (ABI) and an ABI 373A DNA Sequencer. Sequence was analyzed using MacVector software.

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References

Akashi, K., and Weissman, I.L. (1996). The c-kit$^+$ maturation pathway in mouse thymic T cell development: lineages and selection. Immunity *5*, 147–161.

Barthlott, T., Kohler, H., Pircher, H., and Eichmann, K. (1997). Differentiation of CD4^{high}CD8^{low} coreceptor-skewed thymocytes into mature CD8 single-positive cells independent of MHC class I recognition. Eur. J. Immunol. *27*, 2024–2032.

Dudley, E.C., Petrie, H.T., Shah, M.L., Owen, M.J., and Hayday, A.C. (1994). T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. Immunity *1*, 83–93.

Egerton, M., Scollay, R., and Shortman, K. (1990). Kinetics of mature T-cell development in the thymus. Proc. Nat. Acad. Sci. USA *87*, 2579–2582.

Fehling, H.J., and von Boehmer, H. (1997). Early αβ T cell development in the thymus of normal and genetically altered mice. Curr. Opin. Immunol. *9*, 263–275.

Ferguson, S.E., Accavitti, M.A., Wang, D.D., Chen, C.L., and Thompson, C.B. (1994). Regulation of RAG-2 protein expression in avian thymocytes. Mol. Cell. Biol. *14*, 7298–7305.

Goverman, J., Woods, A., Larson, L., Weiner, L.P., Hood, L., and Zaller, D.M. (1993). Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell *72*, 551–560.

Guidos, C.J., Danska, J.S., Fathman, C.G., and Weissman, I.L. (1990). T cell receptor-mediated negative selection of autoreactive T lymphocyte precursors occurs after commitment to the CD4 or CD8 lineages. J. Exp. Med. *172*, 835–845.

Hoffman, E.S., Passoni, L., Crompton, T., Leu, T.M.J., Schatz, D.G., Koff, A., Owen, M.J., and Hayday, A.C. (1996). Productive T cell receptor beta chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev. *10*, 948–962.

Janeway, C.A., Jr. (1994). Thymic selection: two pathways to life and two to death. Immunity *1*, 3–6.

Jorgensen, J.L., Esser, U., Fazekas de St. Groth, B., Reay, P.A., and Davis, M.M. (1992). Mapping T cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. Nature *355*, 224–230.

Kersh, G.J., and Allen, P.M. (1996). Essential flexibility in the T cell recognition of antigen. Nature *380*, 495–498.

Kydd, R, Lundberg, K., Vremec, D., Harris, A.W., and Shortman, K. (1995). Intermediate steps in thymic positive selection: Generation of CD4−8+ T cells in culture from CD4$^+$CD8$^+$, CD4^{int}8$^+$, and CD4$^+$8^{int} thymocytes with up-regulated levels of TCR-CD3. J. Immunol. *155*, 3806–3814.

Lin, W.-C., and Desiderio, S. (1993). Regulation of V(D)J recombination activator protein RAG-2. Science *260,* 953–959.

Lucas, B., Vasseur, F., and Penit, C. (1995). Stochastic coreceptor shut-off is restricted to the CD4 lineage maturation pathway. J. Exp. Med. *181*, 1623–1633.

Lucas, B., and Germain, R.N. (1996). Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4$^+$CD8$^+$ thymocyte differentiation. Immunity *5*, 461–477.

Lundberg, K., and Shortman, K. (1994). Small cortical thymocytes are subject to positive selection. J. Exp. Med. *179*, 1475–1483.

Lundberg, K., Heath, W., Kontgen, F., Carbone, F.R., and Shortman, K. (1995). Intermediate steps in positive selection: differentiation of CD4$^+$8^{int} thymocytes into CD4$^-$8$^+$TCR^{hi} thymocytes. J. Exp. Med. *181*, 1643–1651.

Page, D.M., Kane, L.P., Onami, T.M., and Hedrick, S.M. (1996). Cellular and biochemical requirements for thymocyte negative selection. Semin. Immunol. *8*, 69–82.

Penit, C., and Vasseur, F. (1997). Expansion of mature thymocyte subsets before emigration to the periphery. J. Immunol. *159*, 4848–4856.

Petrie, H.T., Strasser, A., Harris, A.H., Hugo, P., and Shortman, K. (1993). CD4$^+$8$^-$ and CD4$^-$8$^+$ mature thymocytes require different post-selection processing for final development. J. Immunol. *151*, 1273–1279.

Petrie, H.T., Livak, F., Burtrum, D., and Mazel, S. (1995). T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. J. Exp. Med. *182*: 121–127.

Punt, J.A., Suzuki, H., Granger, L.G., Sharrow, S.O., and Singer, A. (1996). Lineage commitment in the thymus: only the most differentiated (TCR^{hi}bcl-2^{hi}) subset of CD4$^+$CD8$^+$ thymocytes has selectively terminated CD4 or CD8 synthesis. J. Exp. Med. *184*, 2091–2099.

Rooke, R., Waltzinger, C., Benoist, C., and Mathis, D. (1997). Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenovirus. Immunity *7*, 123–134.

Sant'Angelo, D.B., Waterbury, G., Preston-Hurlburt, P., Yoon, S.T., Medzhitov, R., Hong, S.-c., and Janeway, C.A., Jr. (1996). The specificity and orientation of a TCR to its peptide-MHC class II ligands. Immunity *4*, 367–376.

Sant'Angelo, D.B., Waterbury, P.G., Cohen, B.E., Martin, W.D., Van Kaer, L., Hayday, A.C., and Janeway, C.A., Jr. (1997). The imprint of intrathymic self-peptides on the mature T cell receptor repertoire. Immunity *7*, 517–524.

Shortman, K., and Wu, L. (1996). Early T lymphocyte progenitors. (1996) Annu. Rev. Immunol. *14*, 29–47.

Suzuki, H., Punt, J.A., Granger, L.G., and Singer, A. (1995). Asymmetric signaling requirements for thymocyte commitment to the CD4$^+$ versus the CD8$^+$ T cell lineages: a new perspective on thymic commitment and selection. Immunity *2*, 413–425.

Swat, W., von Boehmer, H., and Kisielow, P. (1994). Small CD4$^+$CD8$^+$TCR^{low} thymocytes contain precursors of mature T cells. Eur. J. Immonol. *24*, 1010–1012.

Tourigny, M.R., Mazel, S., Burtrum, D.B., and Petrie, H.T. (1997). T cell receptor (TCR) beta gene recombination: dissociation from cell cycle regulation and developmental progression during T cell ontogeny. J. Exp. Med. *185*, 1549–1556.

Urban, J.L., Kumar, V., Kono, D.H., Gomez, C., Horvath, S.J., Clayton, J., Ando, D.G., Sercarz, E.E., and Hood, L. (1998). Restricted use of T cell receptor V genes in murine autoimmune encephalomyelitis raises possibilities for antibody therapy. Cell *54*, 577–592.

van Meerwijk, J.P.M., and Germain, R.N. (1993). Development of mature CD8$^+$ thymocytes: selection rather than instruction? Science *261*, 911–915.

von Boehmer, H. (1994). Positive selection of lymphocytes. Cell *76,* 219–228.